Molecular assembly of multilayer enzyme: toward the development of a chemiluminescence-based fiber optic biosensor

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Abstract

We report here on a technique to immobilize a multilayer enzyme assembly on an optical fiber surface. A multilayer of an enzyme, alkaline phosphatase, was immobilized by chemical cross-linking on an optical fiber surface. Chemiluminescence, ellipsometry, and surface plasmon resonance were used to characterize the structure and activity of the assembly. A chemiluminescence-based fiber optic biosensor utilizing this immobilization technique has been developed for the detection of organophosphorus-based pesticides. Detection of pesticide at sub-ppm level has been achieved for paraoxon.

Keywords: Biosensors; Molecular assembly; Optical fiber sensor; Chemiluminescence; Pesticide

1. Introduction

Biosensors have received considerable attention for both environmental monitoring and clinical diagnosis [1,2]. In particular, biosensors based on optical fiber technology offer significant advantages over conventional sensors because of their remote sensing capability and ease of miniaturization [1,3,4]. A fiber optic sensor uses an optical fiber as a waveguide medium to transmit excitation light to a sensing material and to transmit signals back to the detector. In addition, an optical fiber can also serve as a sensing element by directly immobilizing recognition molecules (e.g., antibodies, enzymes, etc.) on the fiber surface. Immobilization of biomolecules on the surface of a fiber is one of the critical issues for the successful development of fiber optic sensors. A methodology that can effectively immobilize biomolecules onto a fiber surface and yet retain the inherent molecular recognition properties of biomolecules is very desirable for biosensing.

There is considerable public concern over the widespread use of pesticides in agriculture. The organophosphorus (OP)-based insecticides are a class of pesticide that can inhibit acetylcholine esterase, a key enzyme for nerve functions [5]. Their high toxicity and relative higher solubility in water pose a threat to aquatic life unless they are hydrolyzed. Sensors that offer rapid, specific, and sensitive detection are needed to monitor and control pesticide contamination in agriculture runoff and other environments. A number of sensors based on amperometric, chromatographic, and biological methods for the detection of OP-based pesticides have been reported [6–10]. However, the detection speed, sensitivity, and compactness of these sensors still pose a barrier to the wide use of these sensor devices in the field. We have earlier reported a method of using enzyme catalyzed chemiluminescence for the detection of OP-based pesticides. The detection mechanism utilizes the fact that both OP-based pesticides and the chemiluminescent substrate compete for the enzyme, which results in partial inhibition of the chemiluminescence signal. Using alkaline phosphatase and paraoxon as an example, it has been demonstrated that ppb levels OP-based pesticide can be detected in less than 30 s in bulk solution [11]. In this paper, we report the development of a chemiluminescence-based fiber optic sensor for pesticide detection.

A chemiluminescence-based fiber optic sensor has the advantage that no external light source is required. However, the signal intensity from chemiluminescence is usually much weaker than what can be achieved by fluorescence. For the enzyme catalyzed chemiluminescence biosensor, the sensitivity depends on the amounts of enzyme immobilized onto the fiber optic surface. It is therefore important to develop a
methodology that can control and optimize the amount of enzyme immobilized. There are a number of methods available to immobilize biomolecules on an optic fiber. These include molecular adsorption, covalent attachment, and polymer entrapment. Sol–gel entrapment of alkaline phosphatase has also been reported [12–14]. However, it was found that enzyme leaches out due to the high pH of the substrate solution [15]. We report here on a technique to immobilize a multilayer enzyme assembly on the optic fiber surface and the development of a fiber optic chemiluminescence biosensor using this multilayer enzyme assembly. Chemiluminescence, ellipsometry, and surface plasmon resonance are used to characterize the structure and activity of the enzyme assembly. Detection of sub-pm levels of OP-based pesticide using the fiber optic biosensor is demonstrated.

2. Materials and methods

The enzyme alkaline phosphatase, purchased from Sigma Chemicals Co. (St. Louis, MO), was dialyzed in phosphate buffer (pH 7.0) to remove Tris salt before use. Bis(sulfosuccinimidyl) suberate (BS3), a bifunctional crosslinker reagent, was purchased from Pierce Chemical Co. (Rockford, IL) and used without further purification. 3-aminopropyltriethoxysilane and other solvents were supplied by Aldrich Chemical Company. The chemiluminescence substrate solution, Chloro 3-(4-methoxy spiro [1,2-dioxetane-3,2'-tricyclo-[3.3.1.1]decan]-4-yl) phenyl phosphate (CSPD), was purchased from Tropix Inc. (Bedford, MA) as a part of the Southern-Light™ chemiluminescence detection system, which also included diethyiamine (DEA) and Sapphire II, a chemiluminescence amplifying reagent. Paraffin was supplied by Sigma. The multimode step-index optical fiber with diameter of 200 μm was purchased from SpecTran Co (Sturbridge, MA).

Our new immobilization approach involves building a multilayer enzyme assembly on the surface of a glass or silica fiber. Each layer consists of a spacer and coupled enzymes. The spacer is 11.4 Å long and serves as a linker between the enzyme layers. The enzyme is covalently linked to the amino group by a bifunctional amino coupling reagent, Bis(sulfosuccinimidyl) suberate. Fig. 1 shows the schematic diagram of the multilayer enzyme assembly process. About 10 cm of the jacket of a multimode optical fiber was removed and the cladding of the tip was then etched away by hydrofluoric acid (HF, 48% by volume). The fiber tip was then tapered to optimize chemiluminescence signal collection [13]. Optical fibers were then silanized by submerging the tip in a dry solution of 2% 3-aminopropyltriethoxysilane for 1 h, followed by rinsing and washing with toluene and methanol separately. The fiber was then dried over night in an 80 °C oven. To couple one layer of enzyme to the fiber, the silanized fiber was first soaked in solution A which consists of 5 mM of BS3 in phosphate buffer solution (25 mM NaCl, 100 mM phosphate buffer, pH 7.5). The fiber was then washed with phosphate buffer solution and submerged in solution B which consists of 0.5 mg ml⁻¹ of alkaline phosphatase in phosphate buffer solution. The second layer of enzyme was assembled on the first enzyme monolayer by first coupling the spacer using BS3 in solution A followed by coupling the enzyme with solution B. This alternate dipping process was repeated to form the multilayer enzyme assembly on the fiber surface. The number of layers deposited was controlled by the alternative dipping step carried out.

The multilayer structure of the immobilized molecular assembly was characterized by a 633 nm ellipsometer (Rudolph Research Inc.). The samples were prepared by immobilizing multilayer enzymes on a frosted glass slide using the same protocol described for fibers. Five slides with 1 to 5 layers of immobilized alkaline phosphatase were prepared for each experiment. The slides were stored in tris buffer (pH 7.4) at 4 °C, washed with deionized water, and dried with nitrogen gas before measurement.

The enzyme activity of the immobilized multilayer enzyme was characterized by chemiluminescence measurement as shown in Fig. 2. A bundle of 4 fibers with immobilized
enzyme was placed in a sample cell (100 μl capillary). All the fibers were 35 cm long with an 8 cm long tapered tip at their distal ends. Chemiluminescence substrate was injected into the capillary. The chemiluminescence signal was collected and transmitted by the fibers to the CCD array detector. The CCD array was cooled down to 0 °C thermoelctrically to reduce the dark count level. The data acquisition was controlled by a PC. Each point in the raw data collected represents the total number of photons detected by the CCD in unit time interval. In order to plot the total chemiluminescence signal versus time, the raw data is integrated over time. The initial velocity, which was used to characterize the enzyme activity, was calculated in the initial linear portion of the integrated signal.

To demonstrate the application of the optical fiber biosensors, we have used the fiber immobilized with multilayer enzyme to detect trace levels of OP-based pesticide. Paraoxon, as a model analyte, was used in our experiment. The same experimental set-up shown in Fig. 2 was used for the measurement. The pesticide solution, at pre-determined concentration, was mixed with the substrate solution right before each measurement and was injected into the sample cell. The chemiluminescence signal was collected and the initial velocity was calculated as a function of the pesticide concentration.

3. Results and discussion

The multilayer structure of the film was characterized by a number of optical techniques. Ellipsometry, one of the effective techniques to characterize both the thickness and refractive index of a thin film, was used to measure the thickness of the immobilized enzyme layer. Fig. 3 summarizes the experimental results from ellipsometric measurement. There are slight variations in the measurement from spot to spot and the data presented in the figure are the average over the measured spots. The error bars were taken from the standard deviations of all the measurements. The measured refractive index shows a nearly consistent value around 1.475 for all layers with little fluctuation. The thickness of each layer is roughly 85 Å for the first two dipping cycles, and 60 Å for the third. However, the assembly of the multilayer structure seems to saturate after the 3rd dipping cycle. The 4th and 5th dipping cycle only increase the thickness by 15 Å. This saturation behavior in the enzyme assembly is also confirmed by results of the enzyme activity measurements that will be discussed later. The saturation of the assembly process is probably due to the increasing inter-cross-linking among the enzymes when the assembly undergoes more dipping cycles. Another possible explanation is the physical geometry of the enzyme. Alkaline phosphatase has an approximately elliptic geometry with dimensions of 50 × 100 Å. Because of the non-spherical nature of the enzyme, only the first layer has a well-defined layer structure. The molecules assembled in subsequent dipping cycles have an increased randomness in the layer structure that could create more steric hindrance and reduce the accessible sites for the subsequent molecular assembly. However, the exact reason for this saturation is not clear and further investigation is required to clarify the mechanism.

The surface plasmon resonance (SPR) technique was also used to characterize the structure of the enzyme assembly. The multilayer enzyme was assembled on a glass substrate coated with 500 nm of gold. The surface plasmon resonance angle was measured after each layer of alkaline phosphatase was immobilized on the gold surface. It was found that the resonance angle shifted for each layer assembled on the gold surface. This shift of the resonance angle for each layer supports the multilayer structure of the enzyme assembly. The detailed analysis of our SPR experiment has been described elsewhere [15].

Since the amount of enzyme immobilized directly affects the chemiluminescence reaction rate, it is important to conduct chemiluminescence experiments to characterize the multilayer enzyme assembly. Both capillaries and fibers have been used for the study. However, only the results from the fibers will be discussed here. Fig. 4 shows the enzyme activity measured by chemiluminescence signals as a function of the
number of enzyme layers. The results indicate that the total enzyme activity increased from 1 to 3 layers. However, after the third layer, each additional cycle of the dipping process actually causes a decrease of the chemiluminescence signal. The saturation in chemiluminescence above 3 layers is consistent with the results from ellipsometry measurement discussed earlier.

The success of a biosensor depends largely on the stability of the immobilized enzyme. We checked the stability of the immobilized enzyme by repeating the enzyme activity measurement for a number of times over a period of 70 days. Fig. 5 shows the experimental result from an optic fiber immobilized with 2 layers of alkaline phosphatase. The result shows that the chemiluminescence signal measured at 5th and 27th day is very similar, which indicates that the immobilized enzyme is stable for at least 27 days.

In order to demonstrate the potential of the multilayer enzyme assembly for fiber optic sensors, a chemiluminescence-based fiber optic sensor is developed for detection of OP-based pesticides. There are two critical issues in developing a chemiluminescence-based fiber optic sensor: optimum fiber tip geometry and robust enzyme immobilization on the surface of a fiber tip. A number of tapered fiber tips with different geometries have been studied to optimize chemiluminescence collection efficiency. It was found that a combination tapered tip configuration offered the best collection efficiency for chemiluminescence [13]. Several different methods of immobilization, including sol–gel entrapment, have been tested [15]. Our results indicate that the multilayer enzyme assembly technique is an effective and robust immobilization method for chemiluminescence-based fiber optic sensors. In addition, it has the potential to immobilize different kinds of enzymes on a fiber surface for biosensors based on a multi-enzyme system. Fig. 6 shows the results of the paraoxon pesticide detection using a tapered optical fiber immobilized with 2 layers of enzyme, where the initial emission rate of the chemiluminescence signal produced from a 0.4 mM CSPD substrate is plotted versus the pesticide concentration (paraoxon). The inhibition of enzymes by the presence of a small amount of paraoxon can be clearly observed as the decrease of the initial emission rate of the chemiluminescence signal. This result indicates that ppm levels of paraoxon can be detected. Because both chemiluminescent substrate and OP-based pesticide compete for the enzyme, the detection limit can be reduced by increasing the amount of enzyme immobilized on the optic fiber. Fig. 7 shows the chemiluminescence signals for different paraoxon concentrations using optic fiber immobilized with three layers of enzyme. The result shows that sub-ppm level detection for paraoxon can be achieved by using optic fibers immobilized with three layers of enzyme.

4. Conclusions

A novel technique to immobilize multiple layers of an enzyme assembly on an optical fiber surface is developed for a chemiluminescence-based biosensor. This method offers the flexibility of controlling the amount of enzyme on a fiber surface. However, experimental results show that there is a
limit on the number of layers that can be assembled for the enzyme alkaline phosphatase. It is demonstrated that although three assembly cycles significantly increase the chemiluminescence signal, additional assembly after the 3rd cycle does not result in any signal increase. A chemiluminescence-based fiber optic biosensor utilizing this immobilization technique has been developed for the detection of OP-based pesticides. Detection of pesticide at sub-ppm levels has been achieved for paraaxon.

Acknowledgements

The authors acknowledge the support from ARO Grant DAAK 60-93-K-0012, ARO URI Grant DAAL 03-91-G-0064 and ARO Cooperative Grant DAAH04-94-2-0003. ZC is a NRC Research Fellow.

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